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A Robust and Easy Method for Simultaneous Quantitation of Glucose and [6,6-d_2] Glucose in Human Plasma using GC-MS

Introduction

Diabetes mellitus is a severe chronic metabolic disease which has seen an explosive increase worldwide [1]. Insulin therapy is the therapy of choice for diabetes.
The pharmaceutical industry is making great efforts to create insulin analogues with better pharmacodynamic profiles for subcutaneous injection than human insulin.
Insulin analogues have to be investigated according to their pharmacokinetic profiles in clinical trials utilizing glucose tracers. Glucose tracer technology allows the mode of action on the peripheral system and on the liver to be investigated. For human clinical trials stable tracers are needed as the use of radioactive tracers is not allowed in humans. [6,6-d_2]glucose is a good candidate as it is commercially available in adequate quality and at moderate cost.

To simultaneously quantify high abundance glucose and low abundance [6,6-d_2]glucose, the utilization of two internal standards is required, which is not possible, however, when using the widely used aldonitrile pentaacetate derivates. Due to strong fragmentation of pentaacetate derivates in EI-mode, no ion can be used for quantification as a consequence of strong interferences. Aldonitrile pentaacetate cannot therefore be used.

We report a new method capable of simultaneously quantifying glucose and [6,6-d_2]glucose by GC-MS. This method employs two internal standards at approximately the same concentrations as glucose and [6,6-d_2]glucose present in the samples.

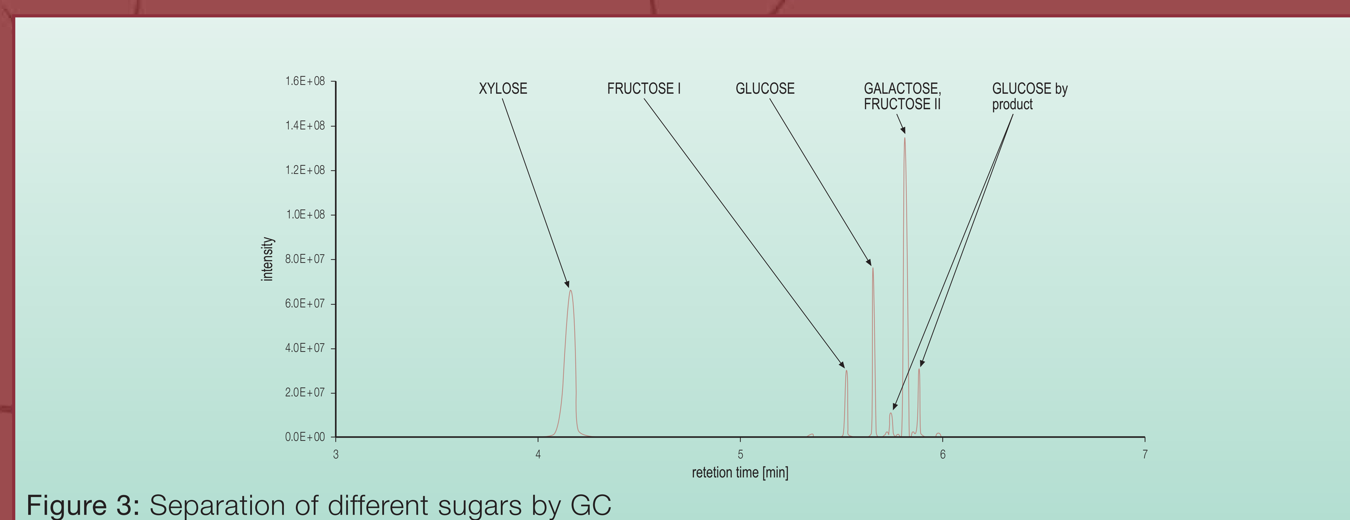

Figure 3: Separation of different sugars by GC

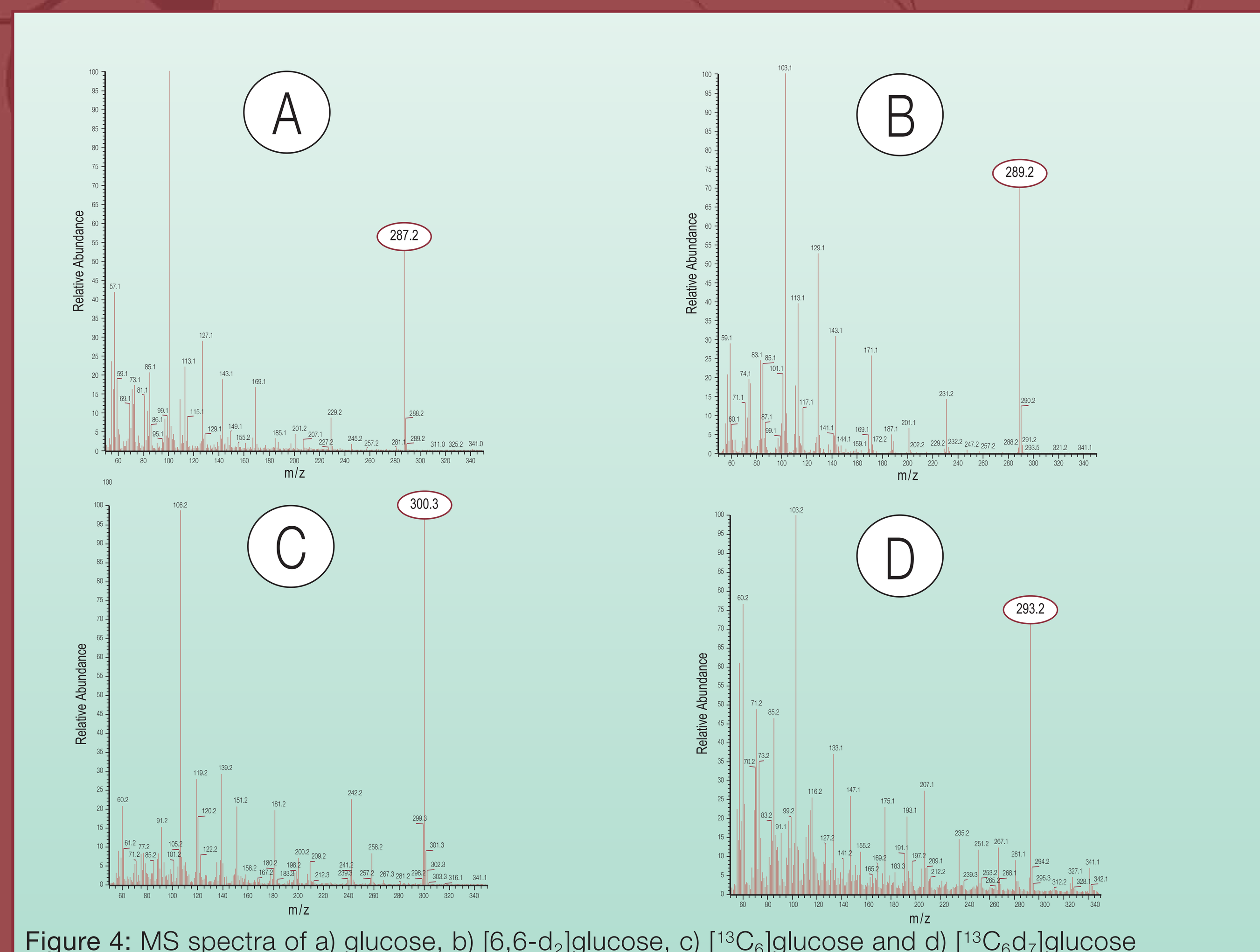

Figure 4: MS spectra of a) glucose, b) [6,6-d_2]glucose, c) [$^{13}C_6$]glucose and d) [$^{13}C_6d_7$]glucose

Methods

Apparatus: Samples were analyzed using a Trace GC-MS (Thermo Finnigan) equipped with an AS 2000 autosampler (Thermo Quest) and Xcalibur software.

GC conditions: GC conditions were as follows: split injection mode (split ratio 11); 5% phenyl methyl siloxane column [DB-5MS; 15 m x 0.25 mm (i.d.); 250 nm film thickness, J&W Scientific, CA, USA]; column temperature program, 1 min at 110 °C, increased by 2.5 °C/min to 118 °C and further increased to 200 °C by 50 °C/min

MS conditions: electron impact mode (EI); source temperature, 200 °C; ionizing potential, 70 e.V.; ionizing current, 150 µamp; traces, m/z 287 (glucose), 289 ([6,6-d_2]glucose), 293 ([$^{13}C_6$]glucose) and 300 ([$^{13}C_6$-d_7]glucose)

Preparation of di-o-isopropylidene acetate (IPAc) derivatives
Derivatization of glucose and [6,6-d_2]glucose was performed according to Hachey et al [2]. Minor changes were made to achieve a method suitable for routine purposes: cold acetone was used for protein precipitation, air was used for all evaporation steps and dichloromethane was used for extraction of di-o-isopropylidene derivatives.

Data analysis
R287/293 and R289/300 were used to quantify d-glucose and [6,6-d_2]glucose. All R289/300 found for [6,6-d_2]glucose were corrected by the contribution factor of natural d-glucose. (R289/300 – R287/293 x R289/287).

Validation
Validation was performed according to ICH and NCCLS guidelines.

[1] K.G. Zimmet, L.G.M.M. Alberti, Nature, 2001, 414, 78
[2] D. L. Hachey, Anal. Chem, 1999, 71, 4734

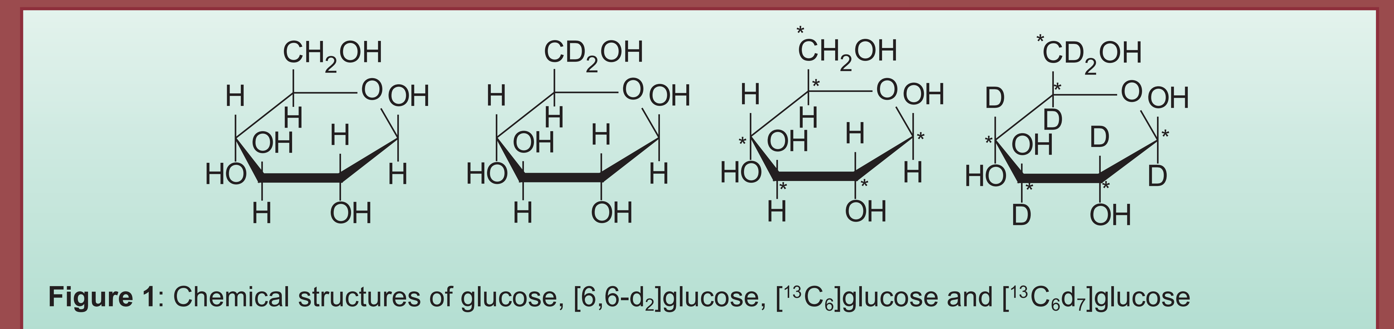

Figure 1: Chemical structures of glucose, [6,6-d_2]glucose, [$^{13}C_6$]glucose and [$^{13}C_6d_7$]glucose

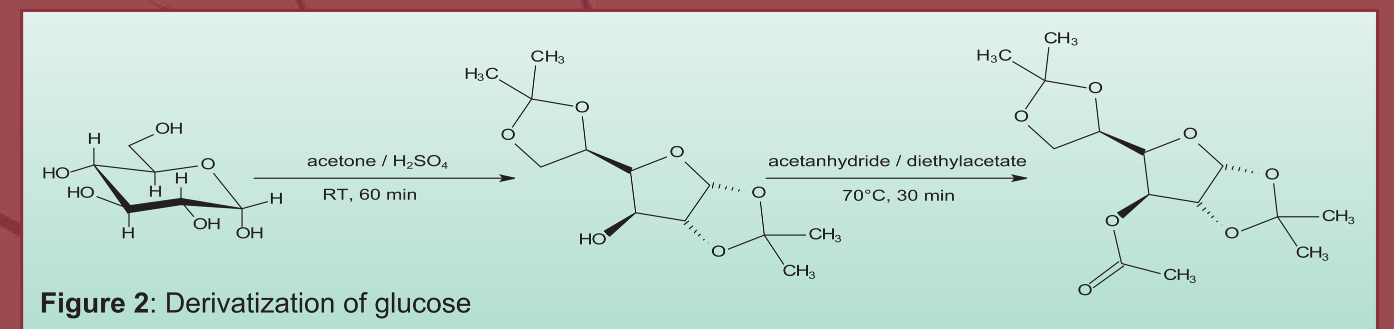

Figure 2: Derivatization of glucose

Results & Discussion

Glusoe isopropyidene derivatives are showing low fragmentation. Therefore, ions with high m/z can be used for quantitaion without interferences.

Linearity (according to NCCLS EP6-P): Calibration curves of d-glucose and [6,6-d_2]glucose are linear. The mean regression equation for d-glucose was: y = -0.0025(±0.0034) + 0.0102x(±0.0000); r^2 = 0.9997; standard error of estimate 0.0190. For [6,6-d_2]glucose: y = -0.0005(±0.004) + 0.3018x(±0.0014); r^2 = 0.9987; standard error of estimate 0.0226.

Precision (according to NCCLS EP5-T): Within-run precision was 1.0% (0.6g/L), 1.1% (1.8g/L) and 0.6% (3g/L) for glucose and 2.4% (12mg/L, 95%), 1.0% (36mg/L, 95%) and 2.1% (60mg/L, 95%) for [6,6-d_2]glucose. Total precision was 1.9% (0.6g/L, 95%), 1.7% (1.8g/L, 95%) and 1.1% (3g/L, 95%) for glucose and 5.2% (12mg/L, 95%), 5% (36mg/L, 95%) and 6.2% (60mg/L, 95%) for [6,6-d_2]glucose.

Accuracy of this method is between 101–104% for glucose and [6,6-d_2]glucose.

Limit of detection is 11mg/L and 0.43mg/L for glucose and [6,6-d_2]glucose, respectively.

Limit of quantification is 38.8mg/L and 1.5mg/L for glucose and [6,6-d_2]glucose, respectively. LoQ and LoD are defined as signal to noise 1:3 and 1:10, respectively.

Specificity was tested for expected components present in human serum samples. D-galactose, D-fructose, D-mannose, D-xylose were tested in regard to interference with D-glucose measurement. No interferences could be observed (see Figure 3). Furthermore, the interference of glucose, [6,6-d_2]glucose, [$^{13}C_6$]glucose, and [$^{13}C_6$-d_7]glucose was investigated. Full spectra of pure standards were recorded and the fragmentation pattern was investigated for potential interferences (see Figure 4). Only natural isotopes of glucose at m+2 could be observed.

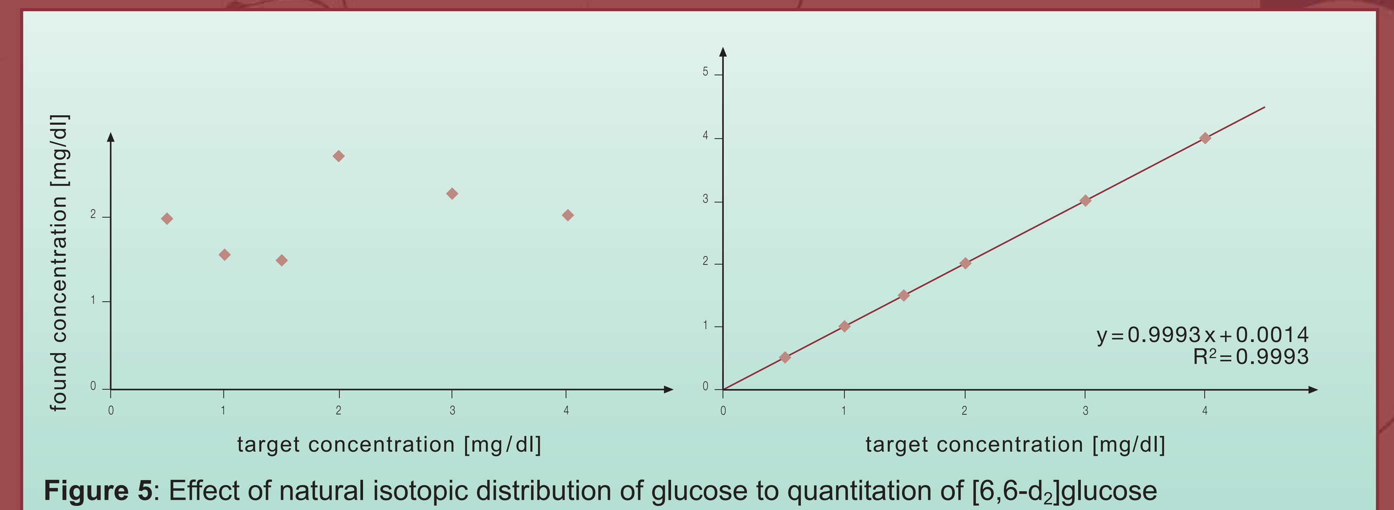

Figure 5: Effect of natural isotopic distribution of glucose to quantitation of [6,6-d_2]glucose

Conclusion

Evaluation of this method showed good performance for the simultaneous determination of glucose and [6,6-d_2]glucose in human sera. By using two internal standards a very robust and accurate method was achieved. This method was employed in several clinical trials and over 5000 samples were analyzed.

Furthermore, by using this method mannitol and [$^{13}C_3$]mannitol can be determined simultaneously with glucose and [6,6-d_2]glucose in human sera. Additionally, [$^{13}C_6$]mannitol must be employed as the internal standard. Mannitol and glucose are detected at the same m/z ratio but are separated by GC, as can be seen in Figure 6.

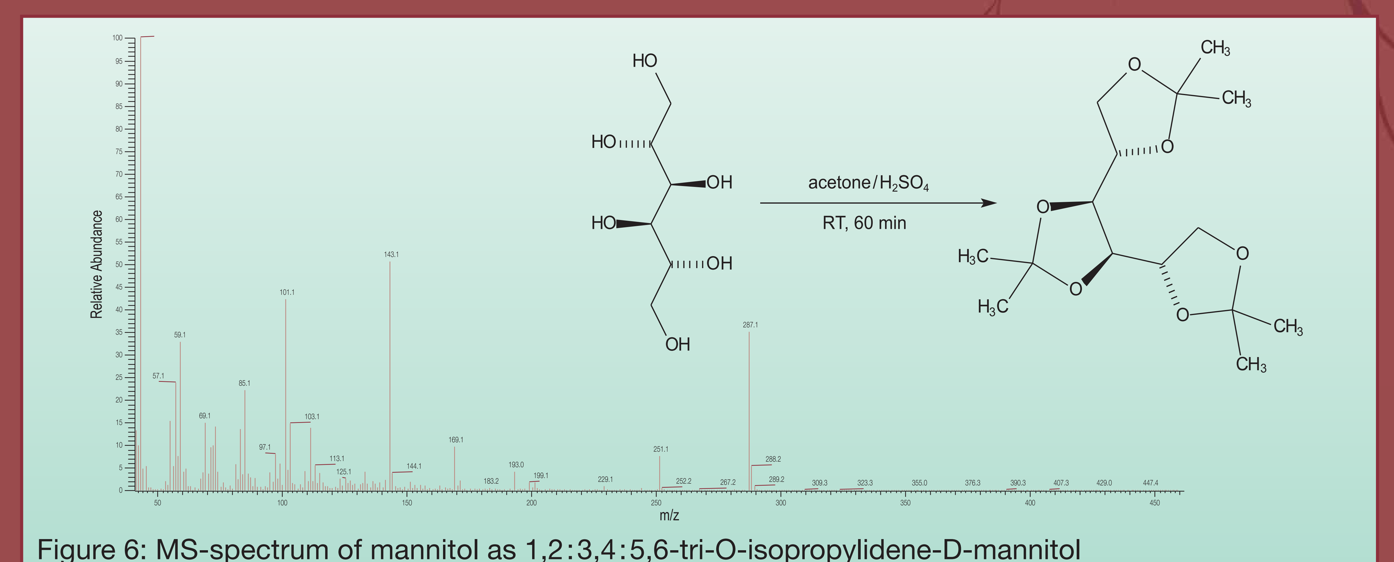

Figure 6: MS-spectrum of mannitol as 1,2:3,4:5,6-tri-O-isopropylidene-D-mannitol

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